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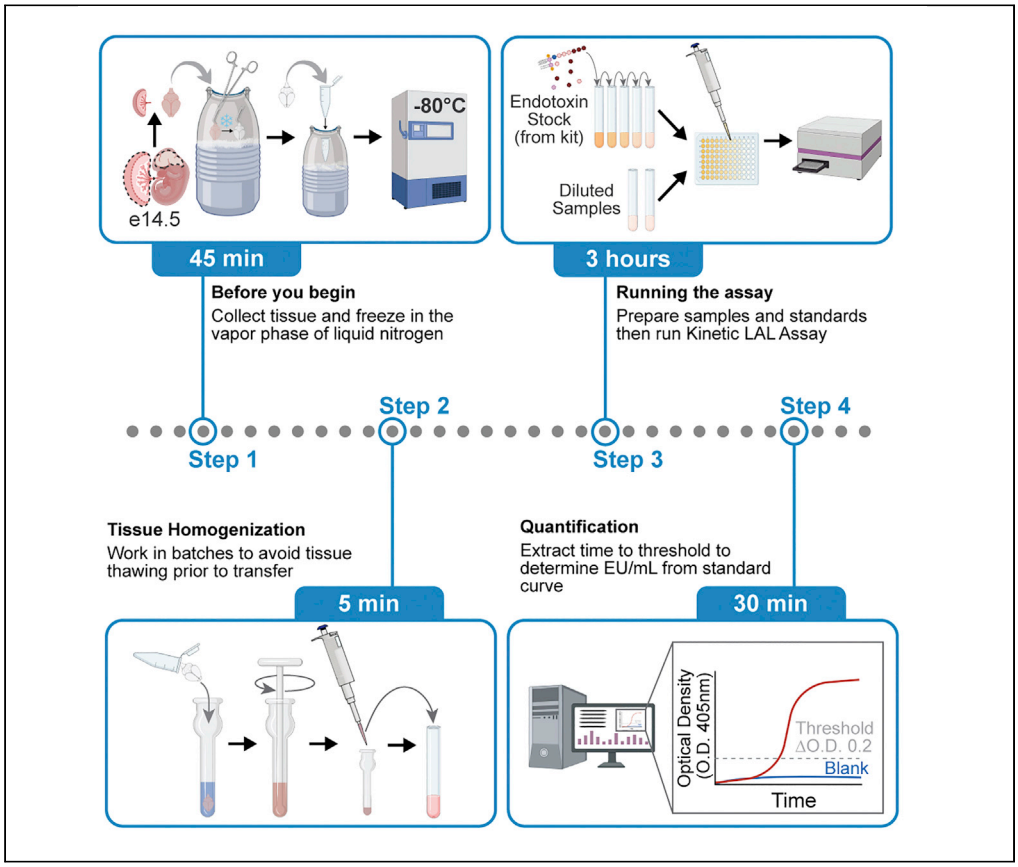

Endotoxin accumulation has been widely noted in several pathologies ranging from metabolic dysregulation to bacterial infection. Using limulus amebocyte lysate (LAL) assays to detect endotoxin load has been the only reliable way to assess endotoxin accumulation, but assays optimized for detection in opaque tissues are still lacking. We optimized a sensitive Kinetic LAL assay for endotoxin detection from murine tissues. In this protocol, we describe tissue collection and homogenization, followed by the procedure to run the assay and data analysis.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol for measuring endotoxin from mouse fetal brain and placenta

Optimization of a Kinetic LAL assay to detect endotoxin in opaque tissues

Step-by-step modifications from commercially available endotoxin detection kit

Details on data analysis and examination of sample inhibition

Ceasrine et al., STAR Protocols 4, 101669
December 16, 2022
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Protocol to measure endotoxin from opaque tissues in mice using an optimized kinetic limulus amebocyte lysate assay

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SUMMARY

Endotoxin accumulation has been widely noted in several pathologies ranging from metabolic dysregulation to bacterial infection. Using limulus amebocyte lysate (LAL) assays to detect endotoxin load has been the only reliable way to assess endotoxin accumulation, but assays optimized for detection in opaque tissues are still lacking. We optimized a sensitive Kinetic LAL assay for endotoxin detection from murine tissues. In this protocol, we describe tissue collection and homogenization, followed by the procedure to run the assay and data analysis.

For complete details on the use and execution of this protocol, please refer to Ceasrine et al. (2022).

BEFORE YOU BEGIN

1. Clean and sterilize all reusable glass materials to remove endotoxin (Sandle, 2013) (dishes, Dounce homogenizers, and tubes if needed). We recommend doing this the day before you begin.
   a. Wash glass materials with dilute Alconox or similar detergent.
   b. Rinse thoroughly (three times with tap water, then 3 times with diH_2O).
   c. Place materials on a few layers of aluminum foil and loosely cover with another layer of aluminum foil. This will allow them to stay covered and sterile once they are removed from the oven.
   d. Bake at 250°C for ≥30 min;
   e. Cool before use.
2. Pre-chill a labeled freezer box in a −80°C for tissue storage. We recommend doing this at least the day before you begin.
3. Set up a template program in your plate reader. This can be done at any point before the day of the assay.
4. This protocol describes the specific steps for using fetal brain and placenta tissue, though other tissues may be used. We also describe analysis using a SPECTROstar Nano plate reader and MARS analysis software from BMG LABTECH Inc., but any kinetic plate reader with temperature control can be used (e.g., BioTek).

Note: Endotoxins leach into plastic – particularly polypropylene. Use glass whenever possible, and polystyrene when glass is not an option. However, we have not found an acceptable 2 mL polystyrene tube for freezing, so we used polypropylene.

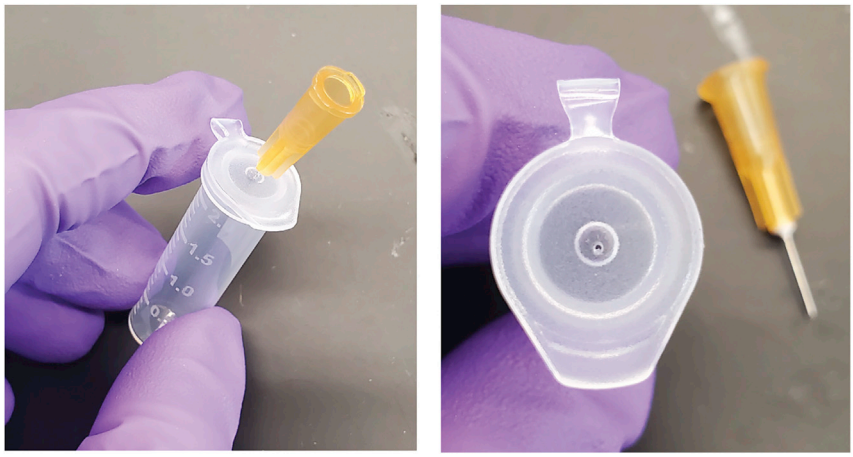

Figure 1. Preparing tubes for freezing samples
Creating a small hole in the tubes used for collection prevents tubes from popping during rapid temperature changes.

Institutional permissions

All procedures relating to animal care and treatment conformed to Duke Institutional Animal Care and Use Committee (IACUC) and NIH guidelines. Before performing animal work, all researchers must obtain approval from their institutions.

Collect tissue

Timing: 45 min/litter

This protocol was optimized using fetal tissue (brain and placenta from embryonic day 14.5 male and female fetuses) from offspring of *C57BL/6J* (Jax: 000664) female mice that had been fed either high-fat diet (45% kcal from fat, Research Diets D12451i) or low-fat diet (10% kcal from fat, Research Diets D12450Hi) for 6 weeks prior to mating with a *C57BL/6J* male mouse. We believe this protocol will work effectively in all mouse strains and with other experimental paradigms.

5. Prepare a sterile environment. There is no need to work in a hood, but you should clean all surfaces with ethanol and allow to dry before starting collections.
   a. Ensure all dissection tools and glass dishes are clean (see "before you begin" step 1).
   b. Poke a small hole (using a sterile 26-31G needle) in the top of each 2 mL polypropylene collection tube (see Figure 1). This will prevent the tube from popping during rapid temperature changes.

   Optional: Saline perfuse pregnant dam prior to embryo collection with cold (stored at 4°C) sterile phosphate buffered saline (PBS; such as Millipore-Sigma TMS-012-A). Keep PBS on ice throughout the perfusion. Perfuse approximately 5 mL of PBS or until the liver has turned from red to light tan.

6. Remove the uterine horns and place on glass dish on ice. Dissect tissue(s) and hold the edge of the tissue with blunt forceps.
7. Hover your tissue in the vapor phase of liquid nitrogen until frozen.

   Note: This should take <1 min/sample.

8. Quickly transfer your sample to 2 mL tube with a hole in the cap (see step 5b and Figure 1). Close the tube and immediately drop it into the liquid nitrogen.

9. Once all samples have been collected, immediately transfer your samples to the pre-chilled box and return it to the −80°C.

Pause point: We have successfully stored tissues for 6+ weeks in −80°C.

Prepare plate reader template

Timing: ~15 min

10. Ensure that your plate reader has a 405 nm filter, and is capable of temperature control.
11. Create a template with the following parameters:
    a. Temperature: 37°C.
    b. Measurement filter: 405 nm.
    c. Kinetic interval: 2 min and 30 s.
    d. Number of reads: 37.
    e. Mixing: 30 s at medium intensity before starting.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
Kinetic-QCL™ Kinetic Chromogenic LAL Assay	Lonza	Cat# 50-650U
Experimental models: Organisms/strains		
C57BL/6J mice (e14.5 male or female fetuses)	The Jackson Laboratory	000664
Software and algorithms		
MARS Data Analysis Software	BMG LABTECH	https://www.bmglabtech.com/en/microplate-reader-software/
Other		
Pyrogen-free Dilution Tubes	Lonza	N207
LAL Reagent Grade™ Multi-well Plates	Lonza	25-340
SPECTROstar Nano Absorbance Plate Reader	BMG LABTECH	https://www.bmglabtech.com/en/spectrostar-nano/
Dounce homogenizer	Fisher Scientific	50-194-5204
Alconox® Powdered Precision Cleaner	Alconox	1104-1
Sterile Phosphate Buffered Saline (PBS)	MilliporeSigma	TMS-012-A
Blunt forceps	VWR	232-0091
Sterile 2 mL tubes	Axygen	MCT-200-C-S

MATERIALS AND EQUIPMENT

All materials should be endotoxin-grade (pyrogen-free). This includes tips (do not use filter tips), 96 well plates, water (LAL Reagent water comes with the kit, but you can order extra separately if needed), and reagent reservoirs (optional, but recommended for running a full plate). Lonza stocks all of these items, but they can also be found from other manufacturers as well. Also make sure that your gloves are powder-free.

Alternatives: We used a SPECTROstar Nano plate reader (BMG) and associated MARS software for analysis, but any kinetic plate reader that can measure absorbance at 405 nm and maintain a temperature of 37°C can be used. Other groups have used Duall Tissue Homogenizers (which are similar to Dounce Homogenizers) in human tissue (Nalepka and Greenfield, 2004).

STEP-BY-STEP METHOD DETAILS

Tissue homogenization

Timing: ~5 min/sample

Fully homogenizing your tissue is critical for assay reproducibility. These steps walk through tissue homogenization and quality control to ensure sample opacity will not interfere with the assay.

1. Place your Dounce homogenizer(s) on ice and add 750 μL Endotoxin free water to each one.

   Note: The amount of water you are homogenizing your sample in should be optimized in house for each sample type. We found 750 μL worked well for ~85 mg of tissue, so this would be a good starting point. However, the properties of each tissue – such as amount of connective tissue - will dictate how much water is needed for thorough homogenization. To run your sample in triplicate (with one replicate being spiked to ensure accurate recovery), a minimum of 300 μL will be needed. To optimize water volume, homogenize one sample in 350 μL endotoxin free water. Add more water (50–100 μL at a time) if needed to ensure tissue is fully homogenized.

2. Transfer your tubes containing your frozen tissue from the −80°C to your workspace on dry ice, and quickly tip your tissue from the tube to the glass homogenizer.

   ⚠ CRITICAL: Do not let your tissue start to thaw in the tube – work in batches if needed!

3. Homogenize your sample manually by twisting and re-inserting the T-shaped pestle into the collection piece of the Dounce homogenizer until there are no visible tissue pieces left.

   ⚠ CRITICAL: Avoid raising the pestle above the tissue suspension volume as this creates bubbles which can make it difficult to see when the tissue is fully homogenized.

   ⚠ CRITICAL: To avoid the tissue suspension spilling out of the homogenizer, be sure the suspension does not exceed one-third the total volume of the chamber.

4. Transfer your tissue homogenate to a sterile glass tube (Pyrogen-free Dilution Tube), and seal well with parafilm and tape (see Figure 2).

   ⏸ Pause point: Once homogenized, your samples can be stored at −20°C for a short period of time. We have stored them for up to one week without issue.

5. Wash and re-sterilize your Dounce homogenizers before preparing another sample (see "before you begin" step 1).

Running the assay

Timing: ~3 h

The following steps are based on the manufacturer's instructions. Briefly, standards are generated by serial dilution for a standard curve, and samples are diluted prior to being loaded onto the plate. Samples and standards are assayed for endotoxin levels [EU/mL].

Note: Plan your plate layout before you begin. While not required, we recommend including a blank (water only) in every run using the water aliquot(s) used to homogenize your tissue. This

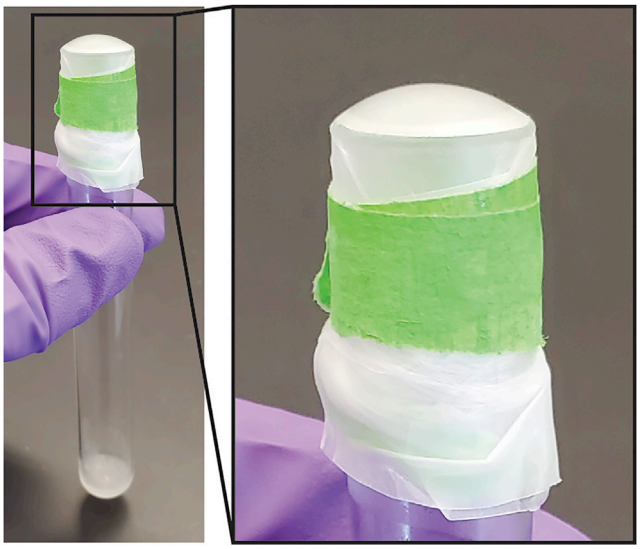

Figure 2. Sealing glass tubes for sample storage
Sealing the tube thoroughly prevents contamination between homogenization and running the assay.

will inform you of any potential contamination within your water (see Figure 4 and Table 1 for expected blank trace/data).

⚠ CRITICAL: Run each sample in triplicate, where one of the triplicates is a spiked sample (see step 11 below). A spiked sample will allow you to ensure that your sample has no intrinsic properties that inhibit the enzymatic activity required for endotoxin detection.

6. Turn the plate reader on and set it to incubate at 37°C.
7. Bring kit reagents to room temperature (20°C–24°C).
8. Prepare standards in sterile glass tubes by serial dilution (see Figure 3). We used the same standard concentrations as recommended in the protocol, but these could be adjusted depending on your sample.
   a. Add 900 µL LAL Reagent water to 4 glass tubes.
   b. Reconstitute the endotoxin standard (*E. coli* 055:B5) in the volume of water specified by your specific kit.

   Note: You can find the volume of water needed for reconstituting your standard at lonza.com/coa. You will need the catalog number and lot number of the kit (not the catalog or lot number from the bottle of endotoxin within the kit). The volume will be listed under "CSE Reconstitution Volume (mL)" on the Certificate of Analysis.

   c. Vortex the reconstituted standard for a minimum of 15 min. This standard is 50.0 Endotoxin Units (EU)/mL.
   d. Add 100 µL of 50.0 EU/mL endotoxin stock to 900 µL LAL Reagent Water in a glass tube. Vortex for at least one minute (This is now your 5.0 EU/mL stock).
   e. Add 100 µL of 5.0 EU/mL endotoxin standard to 900 µL LAL Reagent Water in a glass tube. Vortex for at least one minute (This is now your 0.5 EU/mL stock).
   f. Add 100 µL of 0.5 EU/mL endotoxin standard to 900 µL LAL Reagent Water in a glass tube. Vortex for at least one minute (This is now your 0.05 EU/mL stock).

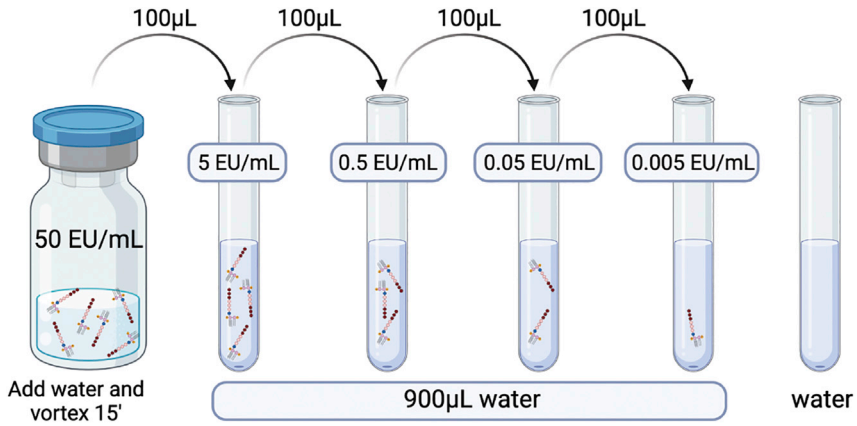

Figure 3. Serial dilution for endotoxin standards

The amount of water needed to add to the lyophilized standard to obtain 50 EU/mL is lot dependent. Figure made with BioRender.

g. Add 100 µL of 0.05 EU/mL endotoxin standard to 900 µL LAL Reagent Water in a glass tube. Vortex for at least one minute (This is now your 0.005 EU/mL stock).

9. Dilute your samples 1:5 in LAL Reagent Water.

⚠ CRITICAL: Dilution factors will need to be optimized for each tissue type to ensure the opacity of the tissue homogenate does not interfere with the assay. We had success with 1:2-1:5 dilution factors for fetal tissue. To optimize dilution factors, measure the absorbance of your undiluted sample at 405 nm. According to the manufacturer, any sample that has an OD >1.5 at 405 nm needs to be diluted before running. However, we have found that samples with an OD >1 can also be less reliable, and we recommend diluting your sample until you reach an OD <1. To do this, add endotoxin free water (50–100 µL at a time), briefly vortex your tissue homogenate, and re-measure the absorbance of your sample at 405 nm. Use the final volume of added water in your test sample for all other samples moving forward and make note of the dilution factor for data analysis. If working with larger tissues, it is possible that the tissue suspension could interfere with the assay in the case of incomplete homogenization. See "Limitations: use of adult tissue" for further optimization.

10. Load 100 µL of your standards and diluted samples to the plate in triplicate.
11. Add 10 µL of 5.0 EU/mL standard (from step 8d) to one replicate of each sample. This sample should give you an endotoxin readout of 0.5 EU/mL within 50%–200% (0.25–1.0 EU/mL).

⚠ CRITICAL: Running a spiked sample is essential when working with tissue, as tissue opacity (among other intrinsic tissue properties) can significantly interfere with endotoxin detection. If your spiked sample returns an [EU/mL] outside the expected range (see Note following Quantification details), do not use any measurements pertaining to that sample in further analyses. With this protocol, we experienced ~5% samples that needed to be excluded due to inaccurate spiked sample recovery.

Note: If you anticipate the endotoxin levels in your samples to be >1 EU/mL, use the 50.0 EU/mL standard to spike your samples, which should give you a final endotoxin concentration of 5.0 EU/mL within 50%–200% (2.5–10 EU/mL).

12. Once all standards and samples are loaded, place your plate in the prewarmed (37°C) plate reader and incubate for 10 min.

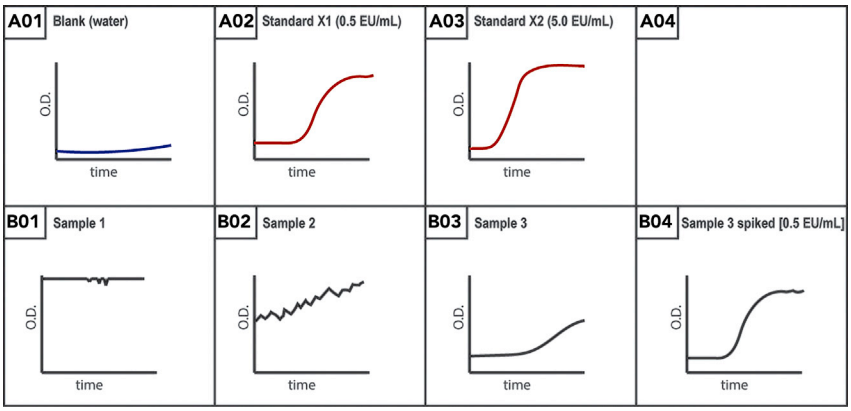

Figure 4. Example trace results of kinetic LAL assay

Optical density (absorbance at 405 nm) versus time is used to determine the 0.2 ΔO.D. threshold (when the absorbance has changed by ≥0.2 compared to the baseline reading). The time to threshold from this baseline corrected data is then used to extrapolate EU/mL from the standard curve. Example graphs in this figure correspond to example data in Table 1.

13. Near the end of the 10-min incubation, reconstitute Kinetic-QCL reagent vial(s) with 2.6 mL LAL Reagent Water/vial.

⚠ CRITICAL: Mix gently – do not vortex.

14. Dispense 100 µL Kinetic-QCL Reagent into all microplate wells and immediately run the Kinetic-QCL program.

⚠ CRITICAL: Do not place lid on the plate!

Note: If working with a lot of samples, use a multichannel pipette and pyrogen-free reservoir (Lonza, Cat# 00190035).

Quantification

Timing: 30 min–1 h

These steps walk through how to analyze the data and check for sample inhibition (when a property of a sample, such as opacity, interferes with endotoxin detection and invalidates the results). For more detail on using MARS software, see: https://www.bmglabtech.com/lonzas-kinetic-kit-for-endotoxin-detection-using-bmg-labtechs-microplate-reader-and-mars-data-analysis/.

15. Save your data and open your run in MARS.
16. Perform a baseline correction for each well based on the raw data (Subtract each measurement from the first measurement).

Note: You can also use the average of the first few cycles as your baseline if your data are variable, although sample variability in early O.D. readings usually indicates that either 1) your sample is too opaque (see example "Sample 1" in Table 1/Figure 1) or 2) your sample was not fully homogenized (see example "Sample 2" in Table 1/Figure 1).

17. Perform a Time to Threshold analysis on your baseline corrected data. Use a threshold of change in optical density (ΔO.D.) 0.2.

Table 1. Example of output data from SPECTROstar nano plate reader and MARS software

Well	Content	Raw data (405)	Baseline correction based on raw data (405)	Time to threshold on baseline corrected (min)	Standard curve linear regression in EU/mL
A01	Blank	0.403	0.301	83.63	0.002
A02	Standard X1 (0.5 EU/mL)	2.733	2.629	21.13	0.572
A03	Standard X2 (5 EU/mL)	2.804	2.699	12.22	5.113
B01	Sample 1	overflow	N.A.	N.A.	N.A.
B01	Sample 2	3.136	0.56	4.82	967.115
B03	Sample 3	2.722	2.27	33.3	0.093
B04	Sample 3 - spiked	3.197	2.747	13.72	3.222

Visualization of kinetic results corresponds to Figure 1.

Note: This will return the time (in minutes) that it took for each well to change by ≥ 0.2.

18. Generate a linear standard curve (Log time (min) to threshold *versus* Log EU/mL).
    a. Sample standard curve: $\log_{10}(Y) = (slope \times \log_{10}(X)) + Y - intercept$

$$Y = \text{Time to Threshold (in seconds).}$$

$$X = \text{EU/mL.}$$

$$\text{Slope} = -0.249998291.$$

$$\text{Y-Intercept} = 3.042474473.$$

19. Extrapolate the EU/mL for each sample from the standard curve.
    a. For Sample 3 (see Table 1 below) the time to threshold is 33.3 min.

$$X = 10\left(\frac{(\log(Y) - Y - intercept)}{slope}\right)$$

$$Y = 33.3 \times 60 = 1998\ seconds$$

$$\log_{10}(Y) = \log_{10}(1998) = 3.300595484$$

$$X = 10\left(\frac{(3.00595484 - 3.042474473)}{-0.249998291}\right)$$

$$X = 0.09279165.$$

20. Average technical replicates.
21. Multiply the extrapolated value by dilution factors as needed.

Note: If your spiked samples do not fall within the accepted recovery range (50%–200%, which is 0.25-1 EU/mL if you used the 5 EU/mL standard to give a 0.5 EU/mL final concentration) you should discard those samples or dilute them further and run them again.

EXPECTED OUTCOMES

Blank (water) values should always fall below 0.005 EU/mL, and our blank values averaged 0.0035 EU/mL. From control e14.5 placenta tissue, we detected 0.0225 EU/mL on average (0.0175 EU/mL from

female and 0.0275 EU/mL from males). From control e14.5 brain tissue, we detected 0.0296 EU/mL (0.0375 EU/mL from female and 0.01917 EU/mL from males) (Ceasrine et al., 2022).

LIMITATIONS

Use in human tissue: We have not attempted to use this protocol in human tissue, although with modifications we believe this protocol could still be applied. Others have demonstrated that β-glucan-like molecules present in human tissue can cause false positives in LAL assays (Roslansky and Novitsky, 1991; Cooper et al., 1997), but the addition of β-glucan blockers can prevent false positives (Nalepka and Greenfield, 2004).

Use of adult tissue: We have successfully used this protocol for murine fetal placenta and brain tissue, and we expect that it would work with other tissues as well, including from adult animals. However, adult tissue, being larger and heavier, will likely be harder to homogenize fully in small enough volumes to detect low-levels of endotoxin and achieve transparent enough input samples. While we have not tested this in-house, we would recommend centrifuging the tissue homogenate immediately after homogenization (2–5 min, 13,000 × *g*, 4°C) to pellet debris that may interfere with the protocol. We recommend centrifuging for as little time as possible to avoid inadvertently pelleting any endotoxin with your debris, and to avoid endotoxin leaching into the tubes. Alternatively, after homogenization you could let your samples sit on ice to naturally pellet any debris, and then transfer the lysate to a clean glass tube and vortexing before loading onto the plate. If these steps are taken, we would recommend treating a set of standards in the same way (i.e., brief centrifugation) to ensure that there is no significant endotoxin loss.

Assay preparation environment: We do not recommend preparing samples for this assay in a hood. While laminar flow hoods/biosafety cabinets create a sterile internal working environment, the increased air flow increases risk of contamination. Instead, we recommend preparing samples on the bench in a clean environment, ensuring no construction is occurring (which may generate unusual air flow/particulates) in the same room.

TROUBLESHOOTING

Problem 1

Tissue is sticking to the tube in which it was frozen.

Potential solution

To avoid this, ensure the tissue is hardened by the vapor phase of the liquid nitrogen before transferring it to the tube (see "collect tissue", steps 7–8). For larger tissues, quickly dipping the tissue in the liquid nitrogen itself will help ensure the outside of the tissue is fully frozen and will not stick to the tube. Further, ensure all tubes are kept on ice (dry ice if available, but regular ice is also OK) prior to transferring tissue. Once the tissue is transferred, quickly shake the tube. If you hear the tissue rattle (i.e., it is not stuck), immediately drop the tube in the liquid nitrogen and gently shake the Dewar to ensure the tube is evenly frozen. If the tissue is already stuck, quickly remove it from the tube and return it to the vapor phase of the liquid nitrogen until it is fully frozen.

Problem 2

Inconsistent results within a plate (i.e., high intra-sample variability between replicates).

Potential solution

- Your tissue was not fully homogenized (see Example "Sample 2" in Table 1/Figure 1). In our hands, Dounce homogenizers worked exceedingly well for tissue homogenization for this protocol. However, all tissues respond to mechanical dissociation differently. See "limitations – Use of adult tissue" for suggestions. We have not tested this assay using anything other than LAL Reagent Water to homogenize tissue, and the inclusion of detergents/other compounds to facilitate tissue

homogenization may interfere with endotoxin detection. Any additional detergents used should be tested for potential interference by running the assay with a blank (solution used to homogenize sample) and spiked blanks.

- Your tissue homogenate is too opaque (see Example "Sample 1" in Table 1/Figure 1). Check the raw baseline O.D. of your samples. If the baseline is too high ($\geq$1 in our experience), dilute and re-run.
- If neither solution 1 nor 2 solves this problem, you can re-analyze the data using the average O.D. from the first few cycles (do not use more than ~5 min worth of readings) as your baseline instead of the singular first reading. However, if the initial issues are not addressed (incomplete homogenization and homogenate opacity), the obtained EU/mL values will likely not be reliable across plates.
- Ensure all reagents/materials (tips, plates, etc.) are pyrogen-free. Do not use filter tips, as the white fibers that make up the filters can interfere with endotoxin detection.

Problem 3

High endotoxin values (>0.005) detected in the blank (water) wells.

Potential solution

- Your glassware was not sufficiently pyrogen-free. Re-sterilize (see "before you begin", step 1) and ensure your oven is dry and reaching the desired temperature for a minimum of 30 min. Increase sterilization time to 45 min.
- Your LAL Reagent Water is contaminated. Replace with a new aliquot.
- Your tubes were not pyrogen-free. Ensure all materials purchased were pyrogen-free and stored in sterile, closed containers.

Problem 4

Endotoxin values are falling outside the standard curve.

Potential solution

- First ensure that your spiked samples are accurate – if they are not, you are experiencing sample inhibition and may need to optimize your homogenization volume or sample pH (see problem 2 and 5).
- If your samples are falling below the standard curve, keep in mind that the limit of detection of this kit is 0.005 EU/mL. Try homogenizing your sample in less water to concentrate the endotoxin in your tissue of interest. You may need to centrifuge your sample if you are not achieving complete homogenization with less water (see problem 2).
- If your samples are above the standard curve (>50 EU/mL), dilute your samples and re-run.

Problem 5

High inter-sample variability within groups.

Potential solution

- Check the pH of your samples and ensure they are all between 6.0 and 8.0. Adjust the pH of any samples outside this range using 0.1 N sodium hydroxide or 0.1 N hydrochloric acid (endotoxin free such as MilliporeSigma 2105-50ML or Fisher Scientific 11-101-5461) and retest your samples.
- Consider using a smaller range for the standard curve (for example, generate 5 standards within 0.005 EU/mL – 25 EU/mL if your sample values are all low). This will increase your precision.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Staci D. Bilbo (staci.bilbo@duke.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate datasets/code.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

A.M.C. and S.D.B. conceived of this study. A.M.C. and L.A.G. performed all experimental optimization steps. A.M.C. performed all data analysis.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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